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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

99113502.1

PRIORITY DOCUMENT

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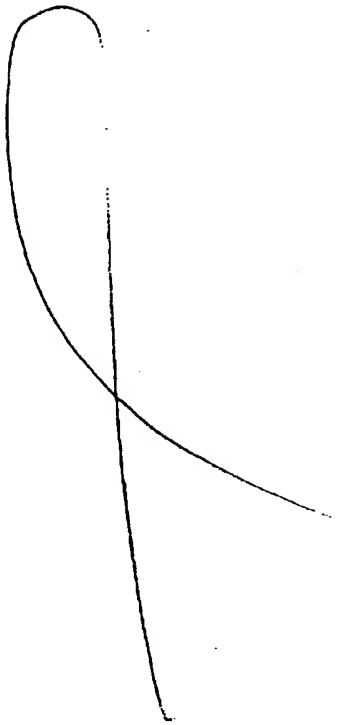
Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 29/05/00
LA HAYE, LE



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Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr
Application no
Demande n°
99113502.1

Anmeldetag
Date of filing
Date de dépôt
02/07/99

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Applicant(s)
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51368 Leverkusen
GERMANY

Bezeichnung der Erfindung
Title of the invention
Titre de l'invention
Angiopoietin-7 and uses thereof

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat State Pays	Tag Date Date	Aktenzeichen File no Numéro de dépôt

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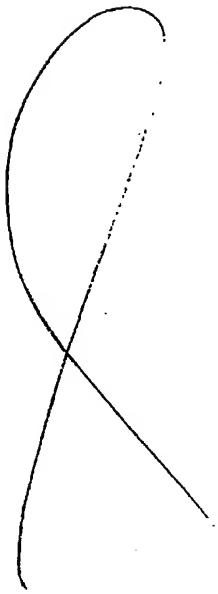
/

Am Anmeldetag benannte Vertragstaaten
Contracting states designated at date of filing AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt

Bemerkungen
Remarks
Remarques



Angiopoietin-7 and uses thereof

The present invention provides a nucleic acid molecule encoding human Angiopoietin-7 (Ang-7) protein. In addition the invention provides methods for producing recombinant human Ang-7 protein. The invention also provides an antibody which specifically binds human Ang-7 protein. The invention further provides for therapeutic compositions as well as a method for modulating angiogenesis.

Introduction

The cellular behavior responsible for the development, maintenance, and repair of differentiated cells and tissues is regulated, in large part, by intercellular signals conveyed via growth factors and similar ligands and their receptors. The receptors are located on the cell surface of responding cells and they bind peptides of polypeptides known as growth factors as well as other hormone-like ligands.

The phosphorylation of tyrosines on proteins by tyrosine kinases is one of the key modes by which signals are transduced across the plasma membrane. Several currently known protein tyrosine kinase genes encode transmembrane receptors for polypeptide growth factors and hormones.

Growth factor receptors of endothelial cells are of particular interest due to the possible involvement of growth factors in several important physiological and pathological processes, such as vasculogenesis, angiogenesis, atherosclerosis, and inflammatory diseases. Also, the receptors of several hematopoietic growth factors are tyrosine kinases.

Receptor tyrosine kinases differ in their specificity and affinity. In general, receptor tyrosine kinases are glycoproteins, which consist of (1) an extracellular domain capable of binding the specific growth factor(s); (2) a transmembrane domain which usually is an alpha-helical portion of the protein; (3) a juxtamembrane domain where

the receptor may be regulated by, e.g., protein phosphorylation; (4) a tyrosine kinase domain which is the enzymatic component of the receptor; and (5) a carboxyterminal tail which in many receptors is involved in recognition and binding of the substrates for the tyrosine kinase.

A gene encoding an endothelial cell transmembrane tyrosine kinase, was described by Partanen, et al., Proc. Natl. Acad. Sci. USA, 87: 8913-8917 (1990). This gene and its encoded protein are called "Tie" which is an abbreviation for "tyrosine kinase with Ig and EGF homology domains." Partanen, et al. Mol. Cell. Biol. 12: 1698-1707 (1992).

Enhanced Tie expression was shown during neovascularization associated with developing ovarian follicles and granulation tissue in skin wounds. Korhonen, et al. Blood 80: 2548-2555 (1992). Thus, Tie has been suggested to play a role in angiogenesis, which is important for developing treatments for solid tumors and several other angiogenesis-dependent diseases such as diabetic retinopathy, psoriasis, atherosclerosis and arthritis.

Two structurally related TIE receptor proteins have been reported to be encoded by distinct genes with related profiles of expression. Both genes were found to be widely expressed in endothelial cells of embryonic and postnatal tissues. Significant levels of Tie-2 transcripts were also present in other embryonic cell populations, including lens epithelium, heart epicardium and regions of mesenchyme. Maisonpierre, et al., Oncogene 8: 1631-1637 (1993).

The predominant expression of the TIE receptor in vascular endothelia suggests that TIE plays a role in the development and maintenance of the vascular system.

Two ligands, angiopoietin-1 (Ang-1) and-2 (Ang-2), have been identified for Tie-2. Angiopoietin-2 is antagonistic to angiopoietin-1, preventing binding of the activating ligand and blocking its ability to stimulate Tie-2 kinase activity and autophos-

phorylation. Angiopoietin-1 and -2 do not bind Tie-1. Angiopoietin-1 and -2 are about 60% identical. They share a similar domain structure with a N-terminal coiled-coil region and a C-terminal fibrinogen-like domain.

Northern analysis shows that angiopoientin-1 is quite widely expressed, but that the expression of angiopoietin-2 is very limited. It is present only in tissues such as ovary, uterus, and placenta, which undergo vascular remodeling.

Recently, two additional ligands for the Tie-2 receptor were identified (Valenzuela et al., Proc. Natl. Acad. Sci. 96: 1904-1909 (1999), Tie ligand-3 (angiopoietin-3 [Ang-3]) and Tie ligand-4 (angiopoietin-4[Ang-4]), but the precise physiological role of Ang 3 and 4 has not been reported yet. However, discovery of new members of the angiopoietin ligand family indicates that other members could exist.

Angiogenesis, concurrent with tissue development and regeneration, depends on the tightly controlled processes of endothelial cell proliferation, migration, differentiation and survival. Dysfunction of the endothelium is a key feature of many diseases including cancer, atherosclerosis, and diabetic angiopathies, to name but a few. Identification of novel Tie receptors and angiopoietins will shed light on the details of how blood vessels are generated, remodelled, and eliminated, thus, providing new tools to improve therapeutic standards to above mentioned indications.

The invention is directed to the Ang-7 protein sequence, corresponding nucleic acid sequences, antibodies, pharmaceuticals compositions, vectors and vector host systems according to the claims.

Ang-7 protein as well as the Ang-7 antibodies can be used in the treatment of diseases as defined above.

Description of the figures.

Fig. 1: Nucleotide sequence encoding human Ang-7.

Fig. 2: Deduced amino-acid sequence of human Ang-7. The sequence is shown in the one letter code of amino-acids.

Fig. 3: Alignment of the amino acid sequences of Ang-1, Ang-2, Ang -3, Ang-4 and Ang-7. Identical amino acids are highlighted by boxes.

Fig. 4: Expression profile of Ang-7.

Fig. 5: In vitro translation of Ang-7. Lane 1: Rainbow [^{14}C]methylated protein molecular weight marker (Amersham, Little Chalfont Buckinghamshire, England) containing following proteins: ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21,5 kDa), lysozyme (14,3 kDa), aprotinin (6,5 kDa). Lane 2: In vitro translation of Ang-7 using the T7 promoter of the mammalian expression vector pcDNA3.1/Myc-His(-) (Invitrogen, Groningen, Netherlands). Lane 3: In vitro translation of Ang-7 using the SP6 promoter of the mammalian expression vector pcDNA3.1/Myc-His(-) (negative control). Lane 4: Positive control from the in vitro translation system (Promega, Madison, USA).

Examples.

Example 1.

With the goal to identify new members of the angiopoietin ligand family a BLAST search (Altschul et al., 1997) of the Expressed Sequence Tag (EST) database from the National Center for Biotechnology Information (NCBI) has been performed. The amino acid sequence of Ang-1 was used as a probe. As a result, a human EST with the accession Number AA773234 was identified. The identified EST showed significant homology to Ang-1 in the reading frame +2. The P value (probability) was $4,4x10^{-28}$ which strongly indicates that the identified EST encodes a fragment of a novel protein which could belong to the family of angiopoietins. Further proof that the newly identified EST encodes a fragment of a novel angiopoietin (lateron designated as Ang-7) was obtained when a BLAST search of a Swissprot database was performed using the identified EST as a probe. The P values obtained for Ang-1 and Ang-2 were $3,2x10^{-32}$ and $2,6 x10^{-34}$, respectively.

Because the commercial EST-clone providers were not able to deliver us the identified EST AA773234, we identified an other homologues EST which belongs to the same gene cluster. The corresponding EST-clone with the accession number AA255590 was purchased and analyzed. Sequencing analysis revealed that the clone AA255590 indeed encodes the fragment of the same gene and includes the sequence of EST AI773234.

Example 2.

For the purpose of full length cloning of the Ang-7 cDNA the HUCL primary membranes (Stratagene, La Jolla, USA) were hybridized with the antisense probe, prepared as described in the example 4. Hybridization revealed a signal at the position L04. The corresponding secondary array membrane was purchased and

hybridized under the same conditions. The signal was detected at the position G19. Thus, the individual clone L04G19 was purchased and analyzed. The clone contained an insert of 2,2 kb. Sequencing analysis confirmed, that this cDNA clone containes the full length coding sequence of Ang-7.

Example 3.

Complete sequencing of the 2173 bp long L04G19 cDNA, which encodes Ang-7 (Seq ID 1, Fig. 1) revealed an open reading frame of 1432 bp which encodes a polypeptide of 493 amino acid residues (Seq. ID 2, Fig. 2). Alignment of the deduced amino acid sequence of Ang-7 with angiopoietins-1, -2, -3 and -4 is shown in Fig. 3. The N-terminal and C-terminal parts of the Ang-7 protein contain characteristic coiled-coil and fibrinogen – like domains, found also in other angiopoietins. The similarity index between the novel Ang-7 and Ang-1 and -2 is 23,9% and 23,5 %, respectively.

Importantly, most of the amino acid residues which are conserved between known angiopoietins are also present in Ang-7 (Fig. 3). This indicates that the identified gene encodes a new member of the angiopoietin family.

Example 4.

To study the expression profile of the identified novel Ang-7, the plasmid AA255590 was linearized with EcoRI and the antisense [^{32}P] radioactively labelled RNA probe was generated using Strip-EZ T3 kit (Ambion, Austin, USA) in accordance to the instructions of the manufacturer. An RNA master blot (Clontech Laboratories, Inc., Palo Alto, CA, USA) was hybridized with the generated probe. The hybridization and washing was performed as recommended in the manual of the Strip-EZ kit. After washing, the membrane was exposed to a phosphorimager screen, scanned on a Fuji Bas-1500 scanner, and the intensity of the radioactive signals was evaluated with the TINA 2.0 software (Raytest, Straubenhardt, Germany). The resulting histogram is

presented on Fig. 4. The novel *ang-7* gene is strongly expressed in heart tissues (atrium left and right, ventricle left and right), uterus, mammary gland and corpus callusum.

Expression of Ang-7 in many tissues encomprising a high circulation of blood, indicates that Ang-7, as Ang-1 and -2, could play a role in angiogenesis.

Example 5.

To test whether the *ang-7* cDNA can be translated to Ang-7 protein and to determine the molecular weight of Ang-7, the complete cDNA was amplified by PCR and cloned upstream of the T7 promoter in the Eco RV and Kpn I restriction sites of the mammalian expression vector pcDNA3.1/Myc-His(-) (Invitrogen, Groningen, Netherlands). For PCR amplification, a 5' primer (5' GCGAATTCACCATGAGGCCACTGTGCGT 3') homologous to the 5' end of the *ang-7* cDNA was used in combination with a 3' primer (5' GGAAGCTTATGGAAGGTGTTGGGGTTCGG 3') homologous to the 3' end of the Ang-7 cDNA. To increase translational efficiency a Kozak consensus sequence was integrated in the 5' primer. For cloning, recognition sequences for the restriction enzymes Eco RV and Kpn I were introduced into the 5'and 3' primers, respectively. The in vitro translation was done essentially according to the instruction of the manufacturer (Promega, Madison,USA) using [^{35}S]methionine. The resulting reaction products were subjected to electrophoresis on a sodium dodecyl sulfate-12% polyarcrylamide gel and visualized by autoradiography.

A major band of ~ 60 kDa was detected (Fig. 5). The observed molecular mass of the major band was slightly larger than the calculated molecular mass of recombinant Ang-7 (~57,1 kDa). However, since the amino acid sequence of Ang-7 contains several potential glycosylation sites the observed larger size of Ang-7 may be produced by incomplete glycosylation of the protein in the in vitro translation system.

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Gln Ser Arg Glu Leu Gln Arg Leu Gln Gly Arg Asn Arg Ala Leu Glu
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Thr Arg Leu Gln Ala Leu Glu Ala Gln His Gln Ala Gln Leu Asn Ser
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Gly Thr Leu Ala Asn Leu Lys His Asn Leu His Ala Leu Ser Ser Asn
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Pro Leu Lys Val Phe Cys Asp Met Glu Thr Asp Gly Gly Gly Trp Thr
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Leu Pro Lys Ser Glu Pro Cys Pro Pro Gly Pro Glu Val Ser Arg Asp
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Asn Thr Gln Val Leu Lys Lys Leu Glu Arg Ala Ile Lys Thr Ile Leu
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Arg Ser Lys Leu Glu Gln Val Gln Gln Gln Met Ala Gln Asn Gln Thr
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Ser Arg Met Asp Ala Gln Met Pro Glu Thr Phe Leu Ser Thr Asn Lys
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His Gln Leu Thr Arg Arg Ala Ala Tyr Ser Leu Arg Val Glu Leu Gln
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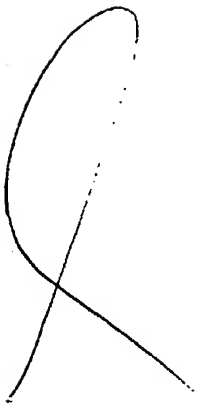

Claims:

1. A polynucleotide comprising a member selected from the group consisting of:

   (a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;

   (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and

   (c) a polynucleotide fragment of the polynucleotide of (a) or (b).

2. The polynucleotide of claim 1 wherein the polynucleotide is DNA.

3. A vector containing one or more of the polynucleotides of claim 1 and 2.

4. A host cell containing the vector of claim 3.

5. A process for producing a polypeptide comprising: expressing from the host cell of claim 4 the polypeptide encoded by said DNA.

6. A polypeptide selected from the group consisting of

   (a) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof, and

   (b) a polypeptide comprising amino acid 1 to amino acid 493 of SEQ ID NO:2.

7. A pharmaceutical composition comprising the polypeptide of claim 6.

8. An antibody capable to bind to the polypeptide of claim 6.

9. Use of the polypeptide of claim 6 for the preparation of medicaments.

10. A diagnostic kit for the detection of the polypeptide of claim 6.

Angiopoietin-7 and uses thereof

Abstract

The present invention provides an nucleic acid molecule encoding human Angiopoietin-7 (Ang-7) protein. In addition the invention provides methods for producing recombinant human Ang-7 protein. The invention also provides an antibody which specifically binds human Ang-7 protein. The invention further provides for therapeutic compositions as well as a method for modulating angiogenesis.

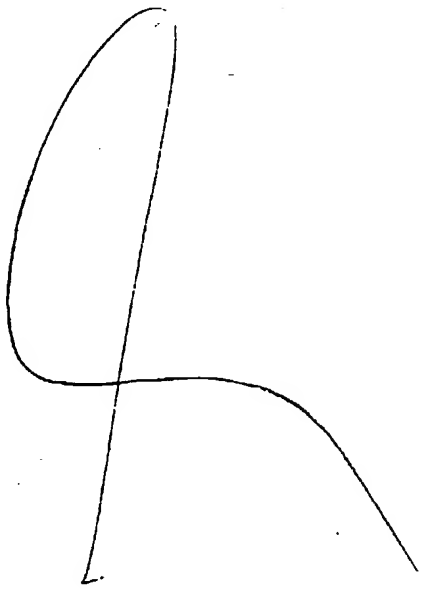

Fig. 1: Sequence ID 1 (Ang-7)

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AGCTGGAGAA CAGGATCCTG AACCAGACAG CCGACATGCT GCAGCTGGCC AGCAAGTACA AGGACCTGGA 910
GCACAAGTAC CAGCACCTGG CCACACTGGC CCACAACCAA TCAGAGATCA TCGCGCAGCT TGAGGAGCAC 980
TGCCAGAGGG TGCCCTCGGC CAGGCCCGTC CCCCAGCCAC CCCCCGCTCC CCGCCCCCGG GTCTACCAAC 1050
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GCTGCCACCC CCTCTGCCCA CTATGCCCAC TCTCACCAGC CTCCCATCTT CCACCGACAA GCCGTCGGGC 1190
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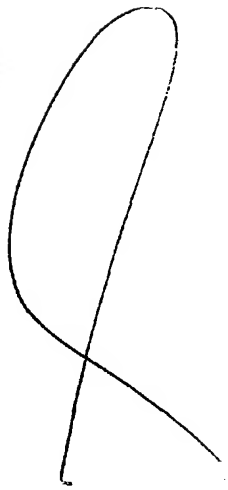

Fig. 2: Sequence ID 2 (Ang-7)

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ALEDGHDTSS IYLVKPENTN RLMQVWCDQR HDPGGWTVIQ RRLDGSVNFF RNWETYKQGF GNIDGEYWLG 350
LENIYWLTNQ GNYKLLVTME DWSGRKVFAE YASFRLEPES EYYKLRLGRY HGNAGDSFTW HNGKQFTTLD 420
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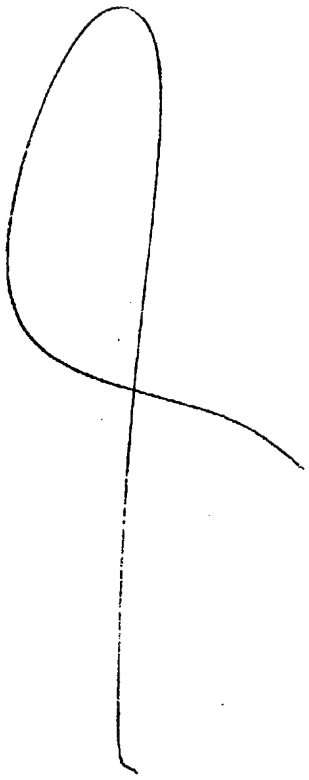
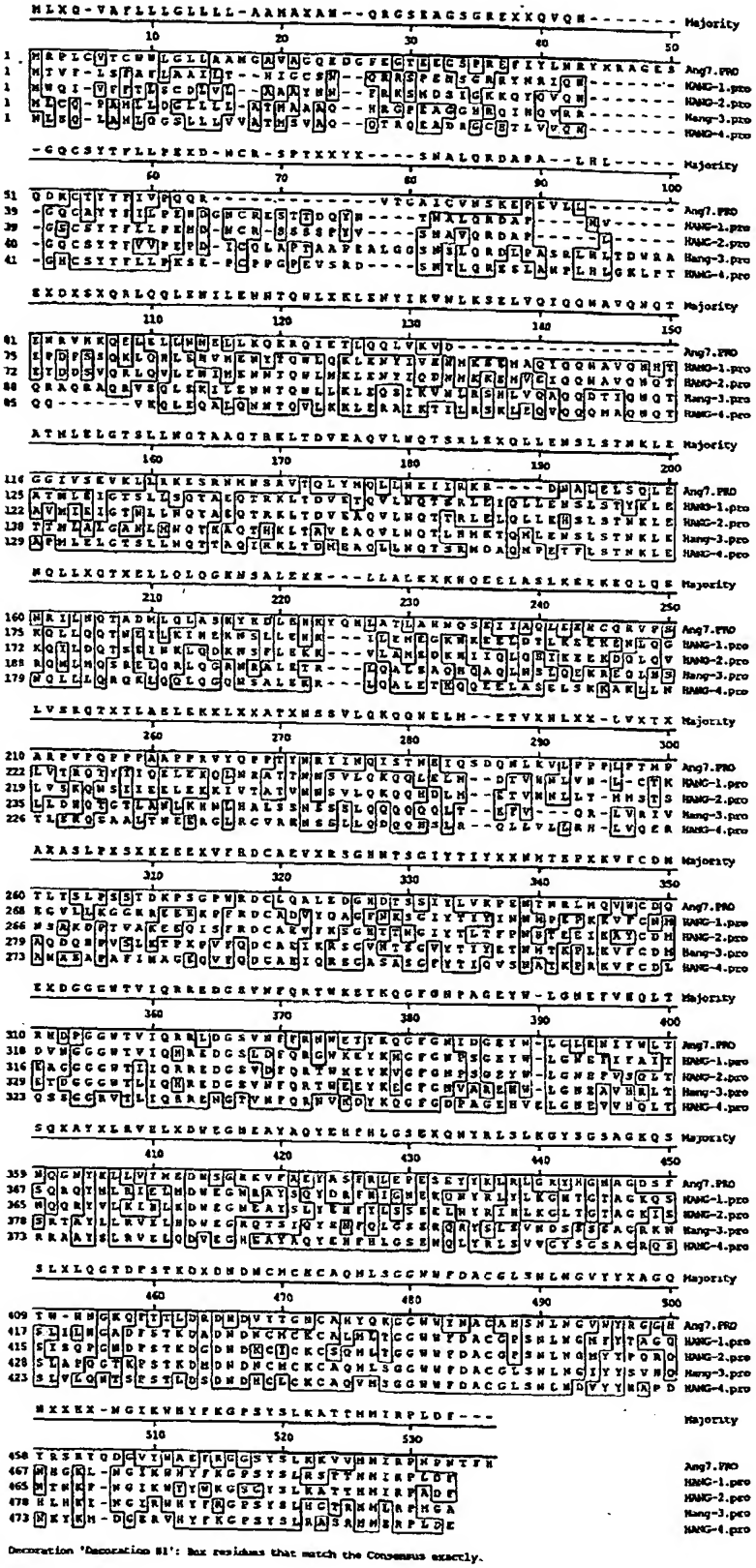
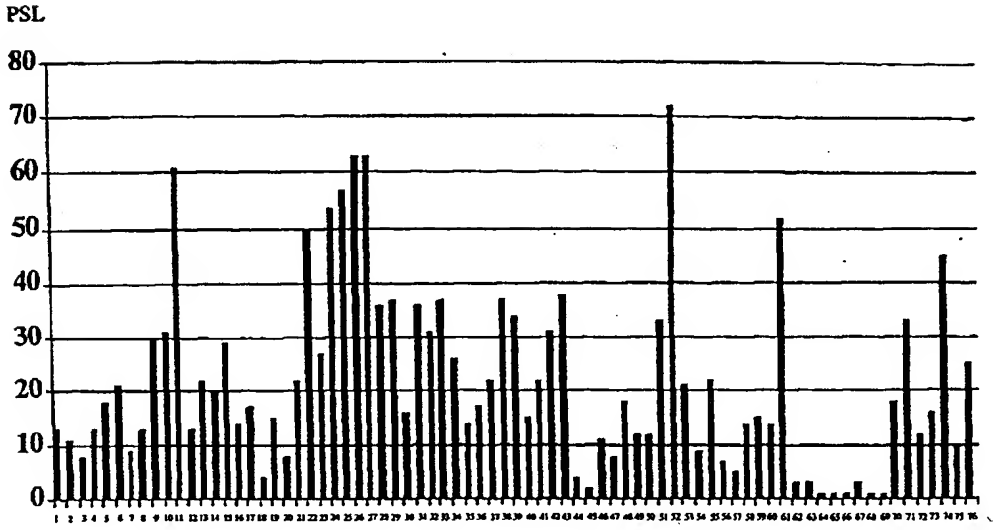


FIGURE 3


Majority
Ang7.PRO
HANG-1.pro
HANG-2.pro
Hang-3.pro
HANG-4.pro
Decoration 'Decoration 81': Box residues that match the Consensus exactly.

Expression profile of Angiopoietin 7



76 different human tissues and cell lines

1- whole brain

2- cerebral cortex

3- frontal lobe

4-parietal lobe

5-occipital lobe

6-temporal lobe

7-paracentral gyrus of cerebral complex

8-pons

9-cerebellum left

10-cerebellum right

11-corpus callusum

12-amygdala

13-caudate nucleus

14-hippocalamus

15-medulla oblongata

16-putamen

17-subtantia nigra

18-accumbens nucleus

19-thalamus

20-pituitary gland

21-spinal cord

22-heart

23-aorta

24-atrium left

25-atrium right

26-ventricle left

27-ventricle right

28-interventricular septum

29-apex of the heart

30-esophagus

31-stomach

32-duodenum

33-jejunum

34-ileum

35-ilocecum

36-appendix

37-colon ascending

38-colon transverse

39-rectum

40-kidney

41-skeletal muscle

42-spleen

43-thymus

44-peripheral blood

45-lymph node

46-bone marrow

47-trachea

48-lung

50-placenta

51-bladder

52-uterus

53-prostate

54-testis

55-ovary

56-liver

57-pancreas

58-adrenal gland

59-thyroid gland

60-salivary gland

61-mammary gland

62-Leukemia HL-60

63-HeLa S3

64-Leukemia K-562

65-Leukemia MOLT-4

66-Burkitt's lymphoma, Raji

67- Burkitt's lymphoma, Daudi

68-colorect. adenocarc. SW-480

69-Lung carcinoma A549

70-fetal brain

71-fetal heart

72-fetal kidney

73-fetal liver

74-fetal spleen

75-fetal thymus

76-fetal lung

Fig.4.

Fig. 5